OsMTN encodes a 5’-methylthioadenosine nucleosidase that is up-regulated during submergence-induced ethylene synthesis in rice (Oryza sativa L.)

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Abstract

Methylthioadenosine (MTA) is released as a by-product of S-adenosylmethionine (AdoMet)-dependent reactions central to ethylene, polyamine, or phytosiderophore biosynthesis. MTA is hydrolysed by methylthioadenosine nucleosidase (MTN; EC 3.2.2.16) into adenine and methylthioribose which is processed through the methionine (Met) cycle to produce a new molecule of AdoMet. In deepwater rice, submergence enhances ethylene biosynthesis, and ethylene in turn influences the methionine cycle through positive feedback regulation of the acireductone dioxygenase gene OsARD1. In rice, MTN is encoded by a single gene designated OsMTN. Recombinant OsMTN enzyme had a $K_M$ for MTA of 2.1 mM and accepted a wide array of 5’ substitutions of the substrate. OsMTN also metabolized S-adenosylhomocysteine (AdoHcy) with 15.9% the rate of MTA. OsMTN transcripts and OsMTN-specific activity increased slowly and in parallel upon submergence, indicating that regulation occurred mainly at the transcriptional level. Neither ethylene, MTA, nor Met regulated OsMTN expression. Analysis of steady-state metabolite levels showed that MTN activity was sufficiently high to prevent Met and AdoMet depletion during long-term ethylene biosynthesis.

Key words: Methylthioadenosine nucleosidase, sulphur metabolism.

Introduction

The methionine (Met) cycle is a metabolic route that helps maintain intracellular levels of Met and S-adenosylmethionine (AdoMet). AdoMet is consumed in the biosynthetic pathways of ethylene (Wang et al., 1982), certain polyamines (Miyazaki and Yang, 1987), and phytosiderophores (Mori and Nishizawa, 1987), producing methylthioadenosine (MTA) as a by-product. MTA, in turn, is a potent inhibitor of the polyamine biosynthetic enzyme spermine synthase (Pegg et al., 1981), of AdoMet-dependent protein methylations such as histone methylation (Woodcock et al., 1983), and of ethylene biosynthesis (Hyodo and Tanaka, 1986).

In plants, the Met cycle was first characterized biochemically (Wang et al., 1982; Miyazaki and Yang, 1987). In recent years, some of the corresponding genes have been identified and characterized (Sauter et al., 2004, 2005; Kobayashi et al., 2005). In plants as well as in most bacteria, the substrate MTA is metabolized by MTA nucleosidase (MTN; Fig. 1). MTN hydrolyses the glycosidic bond between ribose and adenine moieties (Guranowski et al., 1981; Cornell et al., 1996) to release methylthioribose (MTR) and adenine (Ade). In bacteria, this enzyme exerts a second function by hydrolysing AdoHcy (S-adenosylhomocysteine) to S-ribosylhomocysteine (SRH) and Ade (Lee et al., 2003) and is designated MTAN. In plants and in bacteria, MTR is phosphorylated to MTR-1-P (MTR-1-phosphate) by MTR kinase (MTK; EC 2.7.1.100). In contrast, in animals, yeasts, cyanobacteria, and archaea, MTA is converted to MTR-P and Ade by a single
AdoMet is converted to ACC, and MTA is released as rate-limiting step in the biosynthesis of ethylene, where (Mekhedov and Kende, 1996). ACS activity catalyses the genes (Zarembinski and Theologis, 1997; Van der aminocyclopropane-1-carboxylic acid (ACS) through increased transcription of at least two ACC (1-ethylene biosynthesis was shown to be strongly enhanced (Kende et al., 1986). Growth-promoting effects are also seen in species intolerant to hypoxia under defined conditions. For instance, ethylene was shown to promote elongation of the hypocotyl of Arabidopsis seedlings grown in the light (Smal et al., 1997), which contrasts with the better known growth-inhibiting effect of ethylene involved in the triple response of dark-grown Arabidopsis seedlings (Lieberman, 1979).

Growth of semi-aquatic plants in response to ethylene has been particularly well characterized in deepwater rice, in vivo ACS activity was shown to reflect ethylene biosynthetic activity (Cohen and Kende, 1987). Salvage of the methylthioribose moiety of MTA through the methionine cycle replenishes AdoMet. Thus, ethylene can potentially be produced at high rates without interfering with Met or AdoMet homeostasis (Wang et al., 1982).

The aim of the present study was to understand regulation of the Met cycle during enhanced ethylene production. In rice, genes for two enzymes of the Met cycle have been characterized so far. OsARD1, which encodes an acireductone dioxygenase, was up-regulated during submergence in deepwater rice. Gene regulation was shown to be under control of ethylene, indicating that ethylene produced during submergence promoted OsARD1 expression via a positive feedback loop (Sauter et al., 2005). MTR kinase (OsMTK) gene expression, on the other hand, was not elevated during submergence of deepwater rice (Sauter et al., 2004). Pursuing efforts to understand how and at which levels the Met cycle was regulated in relation to ethylene biosynthesis, OsMTN, the unique gene encoding MTN in rice, was identified and characterized.

Materials and methods

Plant material

Seeds of Oryza sativa L. cultivar Pin Gaew 56 were originally obtained from the International Rice Research Institute (IRRI, Los Baños, Philippines). Rice plants were grown as described (Sauter, 1997). For submergence experiments, 10- to 12-week-old plants were submerged in a 600 l plastic tank filled with tap water, leaving about 30 cm of the leaves above the water surface. For MTA and ethephon treatments of stem sections, 20 cm long stem sections containing the youngest internode, cut 2 cm below the second uppermost node, were incubated in 20 ml of 1 mM MTA, 20 ml of 150 µM ethephon, or in the same volume of water for controls. Internodal tissues were harvested as follows, starting above the second highest node: 0–5 mm containing the intercalary meristem, 5–15 mm including the elongation zone, and 30–50 mm representing differentiated tissues (Raskin and Kende, 1984). Freshly harvested tissues were frozen in liquid nitrogen.

Cloning of OsMTN cDNA and overexpression of OsMTN protein

A full-length cDNA encoding OsMTN from rice was isolated through 5′-rapid amplification of cDNA ends from EST C61317, sequenced and deposited in the database under the accession number AF458088. The open-reading frame encoded a protein of 259 amino acids that was named OsMTN (accession number AAL58883). BamHI and NotI restriction sites were adapted by PCR at the 5′ and 3′ end of the full-length OsMTN cDNA, respectively, using the forward primer MTN3/BamHI 5′-ATATTGGATCCGCACGGCCTCTCAGAC-3′ and reverse primer MTN4/NotI 5′-TTAATAGCGGCCATTCAGATCCGATAT-3′. The OsMTN PCR product was cloned into pGEM-T Easy (Promega, Mannheim, Germany) and the BamHI–NotI fragment subsequently shuttled into pGEX-6P-1 (GE Healthcare, Munich, Germany). For expression studies, transformed Escherichia coli BL21 cells were cultured in 250 ml of 2YT medium (Sambrook and
Russel, 2001) supplemented with 100 μg ml⁻¹ ampicillin at 37 °C until the OD600 reached 1.0. Recombinant protein expression was induced for 2 h at 27 °C by incubation in 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were subsequently harvested by centrifugation, lysed by passage through a French press, and soluble recombinant protein released by sonication three times for 15 s each. Lysates were clarified by centrifugation at 30 000 g and 4 °C for 30 min. Recombinant proteins were purified from the supernatant by affinity chromatography on a 4 ml glutathione–Sepharose column according to the manufacturer’s specifications (GE Healthcare, Munich, Germany). On an SDS–polyacrylamide gel, a single protein band of the expected size of 55 kDa was seen when as much as 1 μg of purified recombinant glutathione-S-transferase (GST)–OsMTN fusion protein was separated (data not shown). Purified proteins were aliquoted and stored in 10 mM glutathione, 50 mM TRIS, pH 8.0 at –80 °C. For enzyme analysis, the GST tag was cleaved off using PreScission protease (GE Healthcare, Munich, Germany), and purified OsMTN protein was recovered by filtration on a glutathione–Sepharose column. An E. coli MTAN-specific ELISA (enzyme-linked immunosorbant assay) demonstrated that the purified recombinant OsMTN protein preparation contained no detectable native enzyme derived from the E. coli host cell (data not shown).

RNA isolation and Northern blot hybridization
RNA was extracted from frozen powdered tissues harvested from the youngest internode with Tri-reagent (Sigma-Aldrich, Munich, Germany) following the instructions provided by the manufacturer. A 20 μl aliquot of RNA was separated on a denaturing agarose gel containing 1% (w/v) agarose dissolved in 20 mM MOPS [3-(N-morpholino)-propanesulphonic acid] buffer and 6% (v/v) formaldehyde. Loading of the gels was controlled through ethidium bromide staining of RNA. The RNA filter were performed as described (Sauter, 1997). According to the instructions provided, Hybridization and washing of the RNA filter were performed as described (Sauter, 1997).

Enzyme kinetics
MTN activity was measured using the differential absorbance spectra between the MTA substrate and Ade product. Activity was measured as a decrease in absorbance at 275 nm over time, and the rate calculated using the molar extinction coefficient for MTA of 1.6 mM⁻¹ cm⁻¹ (Singh et al., 2004). Assays were performed using a Cary100 UV/Vis spectrophotometer (Varian, Walnut Creek, CA, USA) with temperature controlled to 22 °C. Reactions contained 990 μl of MTA (0–20 μM) in 50 mM potassium phosphate, pH 7.0, and were initiated by addition of 10 μl of enzyme (1–5 pmol). A decrease in absorbance at 275 nm was monitored for 5–10 min, and initial reaction velocities calculated by fitting the data to a line using WinUV software (Varian, Walnut Creek, CA, USA). The results were fit to the Michaelis–Menten equation using the Cleland enzyme kinetics program (Cleland, 1979).

MTN substrate specificity and specific activity in rice protein extract
To study the substrate specificity of OsMTN, a xanthine oxidase-coupled enzyme assay was used (Lee et al., 2005). This procedure takes advantage of the reactivity of the product Ade with a second enzyme, xanthine oxidase, to form dihydroxyadenine which is capable of oxidizing a weakly coloured tetrazolium salt [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride; INT] to a violet colour that has a strong absorbance in the visible range (470 nm). From the changes in absorbance at 470 nm, the amount of Ade released was calculated using the molar absorption coefficient of 15.4 mM⁻¹ cm⁻¹ at pH 7.0. All substrates were tested at 100 μM, and the rates compared with results obtained with the native substrate MTA. Reactions were mounted in 1 ml containing 50 mM potassium phosphate, pH 7.0, 100 μM MTA, or alternative substrate, 1 mM INT, and 0.25 U of xanthine oxidase grade III from bovine milk (Sigma-Aldrich, Munich, Germany). Enzyme (1–5 pmol) was added, and absorbance at 470 nm was recorded for 5–10 min. The reaction velocity was determined by fitting the data to a line and calculating the initial slope.

To measure OsMTN-specific activity from rice protein extracts, tissue samples from rice plants submerged for the times indicated were ground in liquid nitrogen. Soluble proteins were extracted on ice for 30 min with 50 mM potassium phosphate buffer, pH 7.0. Extracts were centrifuged twice at 30 000 g for 30 min at 4 °C. Protein quantity was measured from the supernatant according to Bradford (1976) using Rotiquant reagent (Roht, Karlsruhe, Germany). A 20 μl aliquot of protein was adjusted to a volume of 20 μl with potassium phosphate buffer and added to 780 μl of the reaction mixture that contained 50 mM potassium phosphate, pH 7.0, 200 μM MTA, 0.2 U of xanthine oxidase grade III from bovine milk, and 1 mM INT (Sigma-Aldrich, Munich, Germany). Initial MTA and Ade levels present in the protein extract were measured from samples without added MTA. Absorbance at 470 nm was measured every 10 min over 2 h with a DU530 spectrophotometer (Beckman Coulter, Krefeld, Germany).

Analysis of metabolites
Metabolites were extracted in 0.5 ml of 0.1 M HCl using 0.2 g of fresh weight of plant material that was powdered in liquid nitrogen. Met was quantified as its fluorescent conjugate of AccQ-Tag after separation by reversed-phase HPLC on a Nova-Pak® C18 3.9×150 mm column (pore size 4 μm). The column was equilibrated with buffer A (140 mM sodium acetate pH 6.3, 7 mM triethanolamine) at a flow rate of 1 ml min⁻¹ and kept at 37 °C. Pure acetonitrile served as buffer B. The gradient was produced by the following concentration changes: 0.5 min 9% B, held for 16 min, 44.5 min 15% B, 47.5 min 60% B, 50.5 min 0% B, re-equilibration for 9.5 min. MTA and AdoMet were determined by spiking of samples prior to extraction with naturally occurring amounts of the respective metabolites. The recovery rates were 83% and 106% with a standard deviation of ~15% for AdoMet and MTA, respectively. The recovery rate of Met was not determined in this study, since extraction of Met from diverse plant tissues by using 0.1 M HCl is routinely used. Key metabolites were identified (i) by spiking of
samples with standards using the chromatographic method described above and (ii) by applying an alternative chromatographic method for separation of AdoMet and MTA in which the gradient of buffer B was changed in order to vary the resolution. No significant differences in the amounts of AdoMet and MTA were observable when both methods were applied.

**Statistical treatment of the data**

Means and standard deviations were calculated and graphically represented for each data point. Student’s *t*-tests were used to compare means and assess statistical significance through calculation of two-tailed probability values (*P*-values).

**Results**

**OsMTN encodes a unique MTA nucleosidase in rice**

In rice, MTN is encoded by a single gene that was cloned and deposited as OsMTN under the accession number AF458088. The open reading frame encodes a protein of 259 amino acids (accession number AAL58883) which showed 25%/51% identity/similarity to MTAN from *E. coli* (Fig. 2). The cDNA of OsMTN was cloned into a bacterial expression vector in-frame with an N-terminal GST in order to express and purify a GST–OsMTN fusion protein in amounts sufficient to perform enzyme assays. After binding onto a GST column, overexpressed protein was purified by elution with glutathione and the GST tag was cleaved by a site-specific protease. The activity of the recombinant rice MTN was subsequently characterized. The kinetic constant was determined using a direct UV absorbance assay according to Singh et al. (2004). Velocities were fit to the Michaelis–Menten equation using the Cleland enzyme kinetics program, and yielded a *K*ₘ for MTA of 2.1±0.2 μM (Fig. 3). The substrate specificity of OsMTN was monitored with a xanthine...
oxidase-coupled assay (Lee et al., 2005). Activity assays with various substrates showed that the plant enzyme accepts a wide array of 5’ substitutions. OsMTN retained 60–80% activity with substrates harbouring short 5’ alkyl-chains such as ethylthioadenosine (ETA), propylthioadenosine (PTA), isobutylthioadenosine (SIBA), and butylthioadenosine (BTA; Table 1). These results largely corroborate those reported by Kushad et al. (1985) who measured MTN activity from tomato extracts. With bulkier substitutions such as phenyl or iodo groups in phenylthioadenosine (PhTA) or 5’-iodoadenosine (IAdo), activities were reduced to ~50%. Addition of charged 5’ cysteinyl or homocysteinyl groups decreased the substrate activity by >80%. Unlike previously published results for lupin (Guranowski et al., 1981), the present results showed that AdoHcy can serve as a substrate for MTN with 16% of the activity obtained with MTA (Table 1).

**Table 1. OsMTN substrate specificity**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Abbreviation</th>
<th>Specific activity (μmol min⁻¹ mg⁻¹)</th>
<th>Maximum activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylthioadenosine</td>
<td>MTA</td>
<td>3.84±0.19</td>
<td>100</td>
</tr>
<tr>
<td>Ethylthioadenosine</td>
<td>ETA</td>
<td>2.95±0.16</td>
<td>76.7</td>
</tr>
<tr>
<td>Isopropylthioadenosine</td>
<td>iPTA</td>
<td>3.04±0.19</td>
<td>79.1</td>
</tr>
<tr>
<td>Propylthioadenosine</td>
<td>PTA</td>
<td>2.85±0.31</td>
<td>74.2</td>
</tr>
<tr>
<td>Isobutylthioadenosine</td>
<td>SIBA</td>
<td>2.75±0.21</td>
<td>71.7</td>
</tr>
<tr>
<td>Butylthioadenosine</td>
<td>BTA</td>
<td>2.35±0.08</td>
<td>61.1</td>
</tr>
<tr>
<td>Phenylthioadenosine</td>
<td>PhTA</td>
<td>2.07±0.32</td>
<td>53.8</td>
</tr>
<tr>
<td>5’-Fluorosulphonylbenzoyladenosine</td>
<td>FSBAdo</td>
<td>0.84±0.07</td>
<td>21.8</td>
</tr>
<tr>
<td>5’-Iodoadenosine</td>
<td>IAdo</td>
<td>2.24±0.09</td>
<td>58.4</td>
</tr>
<tr>
<td>5’-Adenosylcysteine</td>
<td>AdoCys</td>
<td>0.28±0.01</td>
<td>7.27</td>
</tr>
<tr>
<td>5’-Adenosylhomocysteine</td>
<td>AdoHcy</td>
<td>0.61±0.08</td>
<td>15.9</td>
</tr>
</tbody>
</table>
5’ alkylthio-binding pocket. In _E. coli_, this pocket consists of residues from the monomer (Met9, Ile50, and Phe207) and is formed partly by the neighbouring subunit in the homodimer (Pro113, Tyr107, Phe106, and Val102). Only Met9 was strictly conserved. Ile50 and Val102 were replaced by conservative substitutions, valine and isoleucine, respectively. Phe207 was replaced by alanine in OsMTN and either serine, threonine, or alanine in other plant species, while Phe105, Tyr107, and Pro113, which comprise a capping loop donated from the adjacent subunit in the _E. coli_ enzyme, were absent from plant sequences.

**OsMTN expression is induced during submergence**

To see if _OsMTN_ was regulated during submergence, gene expression was monitored in submerged plants through northern blot hybridization. _OsMTN_ transcript levels were measured in the intercalary meristem, in the elongation zone, and in the differentiation zone of growing internodes. The youngest, growing rice internode contains a meristem at the very base that is about 5 mm long. This intercalated meristem produces new cells that are displaced into the elongation zone just above it that extends up to 15 mm in non-submerged and up to 35 mm in submerged plants (Sauter _et al._ 1995). When cells stop elongating, they enter the differentiation zone that extends below the uppermost node and is of variable size depending on the age of the internode.

_OsMTN_ transcript abundance increased between 2 h and 6 h of submergence in the intercalary meristem (Fig. 4A). Minor induction of _OsMTN_ expression was observed after 18 h in the elongation zone. Overall low transcript abundance with no induction by submergence was observed in differentiated tissues. In stem sections treated with the ethylene-releasing compound ethephon, induction of _OsMTN_ gene expression was not observed in a segment encompassing the meristem and part of the elongation zone, indicating that _OsMTN_ was not an ethylene-regulated gene (Fig. 5A).

**OsMTN activity is primarily regulated at the transcriptional level**

To find out if OsMTN was regulated at the protein level, MTN activity was measured in internodes of non-submerged control plants and plants submerged for 2, 4, 6, or 18 h. In non-submerged control plants, MTN activities of 0.59, 0.50, and 0.30 nmol mg^{-1} min^{-1} were measured in the meristem, elongation zone, and differentiation zone, respectively (Fig. 4B). MTN activities in non-submerged plants mirrored _OsMTN_ transcript levels initially present in different tissues (Fig. 4A). MTN activity measured in rice was in agreement with previously reported MTN activity measured in tomato fruits which varied between 0.4 nmol mg^{-1} min^{-1} and 1.2 nmol mg^{-1} min^{-1} depending on the ripening stage (Kushad _et al._, 1985).

As was observed for _OsMTN_ transcript levels, MTN activity was higher in the growing region than in the differentiation zone (Fig. 4A). Activities in the meristem and in the elongation zone were similar to each other at each time point. Between 0 h and 18 h of submergence, MTN activity rose significantly in the meristem (_P_=0.0021) and in the elongation zone (_P_=0.003). Changes in MTN activity in the differentiation zone were not statistically significant (_P_ >0.05). Increased MTN activity during submergence paralleled elevated _OsMTN_ transcript levels. It was therefore concluded that MTN activity was regulated at the transcriptional level.

Since ACC, the immediate precursor of ethylene, is mainly produced in the meristem and in the elongation zone (Cohen and Kende, 1987), regulation of _OsMTN_ gene expression during submergence occurred in the same tissues that produce ethylene during submergence. However, ethylene itself was not the signal that induced either _OsMTN_ expression (Fig. 5A) or _OsMTN_ activity (Fig. 5B) since treatment with the ethylene-releasing compound ethephon did not alter either one within 18 h as compared with controls. Independent experiments confirmed that _OsMTN_ transcript levels were not regulated by ethylene.

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**Fig. 4. OsMTN expression and OsMTN specific activity during submergence.** (A) _OsMTN_ expression was analysed in the intercalary meristem (IM), elongation zone (EZ), and differentiation zone (DZ) of the youngest internode of non-submerged plants (0 h) and of plants submerged for 2, 6, or 18 h. (A) RNA (20 µg) was loaded in each lane. Ethidium bromide-stained rRNA was used to control loading of the RNA. (B) OsMTN enzyme activity was measured in the same tissues as in (A). Assays were performed on 20 µg of total protein for 80 min. Results are averages of five measurements (±SD) obtained from two independent experiments.
Submergence-induced changes in Met, AdoMet, and MTA levels

Unlike OsARD1 which was shown to be an immediate-early ethylene-responsive gene (Sauter et al., 2005), OsMTN was not regulated by ethylene. In yeast, MET genes that encode transcription factors and enzymes responsible for Met and cysteine homeostasis are under control of transcriptional regulation that is sensitive to variations in Met levels (Smothers et al., 2000). To find out more about possible mechanisms of OsMTN regulation, an investigation was carried out to determine if levels of Met, AdoMet, or MTA varied during submergence. Metabolite concentrations were measured in internodal tissues of non-submerged plants and of plants submerged for 2, 6, or 18 h (Fig. 6). Overall, AdoMet and MTA levels increased in all internodal tissues during long-term submergence, although at different absolute values, with the highest amounts present in the meristem. Changes observed after 2 h of submergence were not significant for any of the metabolites measured ($P > 0.05$). In the meristem, AdoMet ($P=0.0033$) and MTA ($P=0.01$) began to accumulate within 6 h of submergence and remained elevated up to 18 h. In the elongation zone, a significant increase was measured for AdoMet ($P=0.011$) after 18 h and for MTA after 6 h ($P=0.008$) and 18 h ($P=0.0033$) as compared with controls. In the differentiation zone, a transient increase in AdoMet ($P=0.036$) and MTA ($P=0.011$) levels was observed after 6 h of submergence. Both AdoMet and MTA returned to basal levels after 18 h.

The distribution of Met in the growing internode differed from that of AdoMet and MTA. Met was present at 2-fold higher levels in the differentiation zone compared with meristem or elongation zone. Overall levels did not change, however, with submergence treatment, with the exception of Met levels in the meristem which were elevated after 18 h as compared with controls ($P=0.032$).
Discussion

The rice MTN is similar to, yet distinct from bacterial MTANs

Animals, yeasts, cyanobacteria, and archaeae use MTA phosphorolase (EC 2.4.2.28) in the Met cycle to convert MTA to MTR-P. Bacteria and plants metabolize MTA in two steps to MTR-P through the intermediate MTR. Bacterial MTANs were described as having a dual function as MTA and AdoHcy nucleosidases which are active in the Met cycle and in the biosynthesis of quorumsensing autoinducer-2 (Miller and Bassler, 2001). The sequence/structure–activity relationship between bacterial MTANs and plant MTNs is well conserved, although plant MTNs possess a leucine in place of a phenylalanine residue in the purine-binding site. While a phenylalanine to alanine mutation at this location in the E. coli MTAN elevated the $K_M$ by 20-fold and reduced the catalytic efficiency to $<1\%$ of the wild-type protein, the rice enzyme maintained MTN activity with a $K_M$ for MTA of 2.1 $\mu$M. Possibly, the predicted essential p–p electron interactions between the adenine purine ring and Phe151 are functionally compensated for in plants by stabilizing hydrophobic interactions with the conserved leucine and/ or H-bond interactions with the adjacent conserved aspartate residue. Other interactions with highly conserved phenylalanine residues in the plant enzyme (Phe140, Phe151, and Phe211) are potentially possible through slightly different folding in the substrate pocket.

Loss of a consecutive stretch of residues conserved in the bacterial MTANs is also interesting. This loop comprising amino acids 105–113 caps the alkylthio-binding pocket and is provided by the adjacent subunit of the dimeric enzyme. In the bacterial protein, Phe105, Tyr107, and Pro113 all form the distal end of a large hydrophobic pocket thought to aid in sequestering the 5′ alkylthio moiety from the solvent and thus improving the stability of the oxacarbenium transition state. Loss of this loop in plant proteins suggests that the adjacent monomer in the plant enzyme does not contribute to the binding of substrate. The reduced depth of the pocket may help explain the decreased activity of OsMTN for substrates bearing larger 5′ substitutions (FSBAdo, AdoCys, and AdoHcy).

Involvement of MTN in AdoHcy hydrolysis might be relevant in the case of limited AdoHcy hydrolase activity

Plants possess dedicated AdoHcy hydrolases, also called adenosylhomocysteinases, to catalyse AdoHcy breakdown (Guranowski and Pawelkiewicz, 1977; Stepkowski et al., 2005). Database searches revealed the existence of a single gene encoding AdoHcy hydrolase in the rice genome (Os11g26850) and two in Arabidopsis (At4g13940 and At5g23810). Previous work reported that MTN purified from lupin (Lupinus luteus) seeds did not accept AdoHcy as a substrate (Guranowski et al., 1981). Using recombinant protein, it was shown here that rice OsMTN could hydrolyse substrates with a wide range of 5′ substitutions. In particular, AdoHcy was hydrolysed with 16% efficiency as compared with MTA. MTAN from Escherichia coli showed 40% activity efficiency with AdoHcy as substrate (Della Ragione et al., 1985). Hydrolysis of AdoHcy by MTN might be a remnant of the activity of an ancestral enzyme from which plant MTNs and bacterial MTANs evolved. Whether AdoHcy hydrolase activity of MTN is also conserved in other plant species remains to be determined. Another major question is whether the AdoHcy hydrolase activity measured in vitro at high substrate concentrations will occur in planta since AdoHcy is maintained in plants at relatively low levels (Moffatt et al., 2002). It is a by-product of AdoMet-dependent methylation and is metabolized to Ado and Hcy by AdoHcy hydrolase (Fig. 1).

The reaction catalysed by AdoHcy hydrolase purified from lupin seeds was reversible and the net reaction measured in vitro was that of AdoHcy synthesis, which was estimated to be 60 times faster than AdoHcy hydrolysis (Guranowski and Pawelkiewicz, 1977). AdoHcy hydrolase activity was affected by the presence of inhibitors such as AdoMet, Hcy, and Ade (Poulton and Butt, 1976). MTN releases Ade during hydrolysis of MTA and may therefore contribute to AdoHcy hydrolase inhibition. However, it is unlikely that Ade levels in vivo reach the inhibitory concentrations required in the in vitro assay performed by Poulton and Butt (1976). On the other hand, MTA itself was reported to inhibit AdoHcy hydrolase in bovine liver (Patel-Thombre and Borchardt, 1985), which suggests that metabolic fluxes towards MTA-producing reactions and MTA catabolites might be accompanied by reduced AdoHcy hydrolase activity. To test this hypothesis in internodal tissues of submerged deepwater rice plants, it would be important to measure (i) the affinity of the rice MTN for AdoHcy; (ii) AdoHcy levels; and (iii) AdoHcy hydrolase activity. Like MTN, AdoHcy hydrolase is encoded by a single gene in rice. Expression analysis of both genes coupled with metabolic profiles could help uncover the contribution of MTN in AdoHcy depurination in planta.

OsMTN is up-regulated concomitantly with increased ethylene biosynthesis but is not regulated by ethylene

In deepwater rice, ethylene is produced in the youngest internode. Upon submergence, ethylene synthesis was shown to be increased through elevated ACS activity in the meristematic and elongation zones, whereas ACS activity remained low in differentiated cells (Cohen and Kende, 1987). Accordingly, OsMTN transcripts accumulated in the intercalary meristem and in the elongation zone, but not in the differentiation zone. Thus both spatial
distribution and temporal changes in OsMTN expression in the internode correlated with ACS activity. One should keep in mind that polyamine biosynthesis is also activated in submerged deepwater rice (Cohen and Kende, 1986). AdoMet decarboxylase activity produces decarboxylated AdoMet that is used for spermine and spermidine synthesis, releasing MTA as a by-product. AdoMet decarboxylase activity was shown to increase upon submergence in the meristem but not in the elongation zone (Cohen and Kende, 1986). OsMTN transcript levels and enzyme activity were elevated not only in the meristem but also in the elongation zone after 18 h of submergence, indicating that regulation of OsMTN expression during submergence might be coupled to ethylene rather than to polyamine biosynthesis.

Induction of OsARD1 expression occurred within 2 h of submergence when ethylene has accumulated to physiologically relevant levels (Sauter et al., 2005). OsMTN transcript accumulation occurred later between 2 h and 6 h, which argues against a direct regulation through ethylene signalling. In fact, treatment with ethylene did not induce OsMTN expression in stem sections, confirming that ethylene was not a signal for OsMTN gene regulation. OsMTN transcript or MTN activity levels were not altered when rice stem sections or suspension-cultured rice cells were incubated in the presence of Met or MTA (data not shown). Thus, the nature of the signal inducing OsMTN during submergence remains elusive. In summary, it can be said that Met cycle genes are not coordinately regulated during submergence (Sauter et al., 2004, 2005; this study). Rather, regulation appears to be exerted by a combination of signals.

OsMTN might support long-term ethylene biosynthesis

One of the functions of the Met cycle is to maintain Met pools during sustained ethylene biosynthesis (Wang et al., 1982). ACS activity in deepwater rice increased about 2-fold within 2 h of submergence and was elevated over controls about 4-fold after 20 h of submergence (Cohen and Kende, 1987). It was shown here that levels of AdoMet, Met, and MTA did not change substantially within the first 2 h of submergence; neither did OsMTN transcript levels nor MTN activity. Taken together, these results indicated that the basal activity of OsMTN was high enough to metabolize MTA released during early ethylene synthesis.

When rice plants were submerged for longer periods, not only ethylene biosynthetic rates, but also MTN activity increased. Concomitantly, steady-state levels of Met, AdoMet, and MTA increased, possibly indicating that the plant adapted to long-term ethylene biosynthesis by up-regulation of the Met cycle including elevation of Met cycle intermediates. This adaptation may be advantageous for long-term ethylene production as was described for submerged deepwater rice which produces ethylene for many days at levels up to 50 times higher than in unsubmerged plants (Métraux and Kende, 1983). This conclusion would fit the observation that most of the changes observed in metabolite amounts and MTN activity occurred in the growing region where ethylene biosynthetic rates are highest (Cohen and Kende, 1987).

Surprisingly, for Met, the highest levels were measured in the differentiation zone. This observation might be partly explained by a reduced flux of Met into AdoMet synthesis or decreased rates of protein biosynthesis in non-growing cells. In general, changes in AdoMet and MTA show a high degree of correlation. Even though this can be taken as a result of the substrate–product relationship between AdoMet and MTA, the result is surprising in view of the fact that AdoMet can be metabolized by enzymes that do not produce MTA and can also be synthesized through pathways other than the Met cycle. For Met, no tight correlation was observed with either AdoMet or MTA, reflecting that Met can be produced through several pathways (Fig. 1) including de novo biosynthesis. The regulatory mechanisms that control these steady-state sulphur metabolite levels remain to be uncovered.

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References


